ISSN (print) 0093-4666 © 2011. Mycotaxon, Ltd. ISSN (online) 2154-8889

MYCOTAXON

Volume 116, pp. 355–364 *April–June 2011*

DOI: 10.5248/116.355

AFLP molecular characterizations of some *Saccobolus* species (*Ascomycota, Pezizales*)

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Abstract — Amplified restriction fragment length polymorphisms (AFLP) were used to assess the genetic diversity among species and isolates of the genus *Saccobolus*. Monosporic strains of five *Saccobolus* species were used throughout. The dendrogram obtained from the analysis of grouping (UPGMA) showed four groups of species. The ordination of isolates through the principal coordinates method exhibited nearly the same relations between isolates as the phenogram. Analysis of six samples identified as *Saccobolus versicolor* using morphological characters indicated the same diagnostic fingerprints as *S. verrucisporus* with the primer combinations tested. The isolates identified as *S. versicolor* may represent an intraspecific variant of *S. verrucisporus*. The results support the use of AFLP markers to delimit *Saccobolus* species. This methodology constitutes an additional tool to study the taxonomy of the genus, which has previously used only morphological characters.

Key words — molecular markers, coprophilous fungi, systematic

Introduction

The genus *Saccobolus* (*Pezizales*, *Ascobolaceae*) was established by Boudier (1869). The presence of a common hyaline sheath around the ascospores was considered one of the major criteria. Brummelen (1967) characterized the genus within the subfamily *Ascoboloideae* as producing clustered ascospores, comparatively short and broad, clavate, truncate asci, and rather short paraphyses.

Saccobolus species are coprophilous fungi. They have a cosmopolitan distribution and their spores usually are transported over long distances.

Most of these fungi are homothallic, probably originated as an adaptation to the substrate, which determines their sexual isolation (Wicklow 1981).

Sexual reproduction enables ascospores to be dispersed by effective discharge methods.

Different concepts have been used to define the fungal species. The phenotypic concept is the classic approach based on morphological characters. The polythetic concept circumscribes species based on a combination of characters (Kohn 1992, Guarro et al. 1999). *Saccobolus* species have traditionally been classified based on cytological and morphological characters (Brummelen 1967) and supported by the phenotypic concept. Even though such characters as ascal structure and dimensions, type of dehiscence, spore ornamentation, and the general ascocarp developmental pattern are clearly important to the classification of the *Saccobolus* species, identification is often uncertain because these characteristics are shared by two or more species or may vary according to culture conditions. Here use of the polythetic concept could prove helpful in characterizing the genus.

In an earlier study, Ramos et al. (2000) performed an isozyme analysis to confirm morphology-based identification of *Saccobolus* species and to detect intraspecific variability. In that study, isozymes showed little intraspecific variability and confirmed the previous identification of the strains using morphological characters, although only few species-specific bands were found.

Molecular techniques have proved to be the appropriate tools to advance in the study of genetic diversity of the intra- and inter-specific variation allowing an insight on the phylogenetic relationships

For instance, the amplified fragment length polymorphism (AFLP), (Vos et al. 1995) is a DNA-based fingerprinting technique frequently used in a diverse array of organisms (Briard et al. 2000, Roa et al. 1997, Zeller et al. 2000) that requires no prior sequence information on the organism under scrutiny.

Although the morphological, physiological, and biochemical characterizations of *Saccobolus* species have been the aim of many studies, molecular study is still being developed.

In the present study we used the AFLP methodology to characterize five *Saccobolus* species so as to investigate the efficiency of these molecular markers in determining genetic diversity, to obtain diagnostic bands for species recognition, and to compare them with the results of a previous isozyme study.

Material & methods

Monosporic strains

Mature apothecia of *Saccobolus citrinus* Boud. & Torrend (Boudier & Torrend 1911), *S. saccoboloides* (Seaver) Brumm. (Brummelen 1967), *S. pseudodepauperatus* Gamundí & Ranalli (Ranalli & Gamundí 1976), *S. verrucisporus* Brumm. (Brummelen 1967), and *S. versicolor* (P. Karst.) P. Karst. (Karsten 1885) were obtained by placing cow and horse

dung from different geographical locations in Petri dishes with a layer of filter paper. Thirty-two monosporic isolates of five species of the genus *Saccobolus* were obtained from individual ascospore germination. Table 1 lists the monosporic isolates of each species with their geographical location, substrate, and BAFC culture collection number. All strains were deposited in the Herbarium and Culture Collection of the Departmento de Biodiversidad, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (BAFC). Cultures of all monosporic strains were regularly kept in PF medium (yeast extract 3 g; agar 18 g; distilled water 1000 ml; a slice of filter paper) (Ranalli & Forchiassin 1974) at 5°C.

Table 1. List of *Saccobolus* isolates employed in this survey with their geographical location and BAFC number.

Species	Locality (Argentina)	Monosporic isolates (BAFC numbers)
S. verrucisporus	Gualeguaychú, ER	3403–3405, 3449, 3450
S. verrucisporus	Gobernador Castro, BA	3410–3412, 3451
S. versicolor	Gualeguaychú, ER	3407–3409, 3452, 3453
S. saccoboloides	Pergamino, BA	3454–3460
S. citrinus	V. Tuerto, SF	3461–3463
S. pseudodepauperatus	Arrecifes, BA	3464–3471

BA = Buenos Aires Province; ER = Entre Ríos Province; SF = Santa Fe Province.

Identification of species

Morphological and cultural studies were carried out in order to identify the species. The characters used to identify the *Saccobolus s*pecies were the same used by Brummelen (1967) that included diverse characteristics of apothecia, ascospores, asci, and paraphyses. Also used were the distribution and appearance of apothecia in PF medium (yeast extract 3 g; agar 18 g; distilled water 1000 ml; a slice of filter paper; Ranalli & Forchiassin 1974) and ascospore germination in AA medium (Bacto agar 20g; distilled water 1000 ml). Studies of spore germination and ascocarp development were performed on AA plates and in PF medium plates respectively.

DNA extraction

Mycelium was obtained according to Ramos et al. (2000) procedure and ground to powder in liquid nitrogen using a sterile pestle. Genomic DNA was extracted following Gottlieb & Lichtwardt (2001). Quality control and quantification of genomic DNA was carried out by agarose gel (0.8% w/v) electrophoresis and by comparison with a DNA molecular-size standard (Lambda EcoRI/HinDIII, Promega Corp.). Ethidium bromide gels were photographed under UV light.

AFLP

The AFLP methodology was carried out on 250 ng of genomic DNA using the AFLP® Analysis System for Microorganisms Primer Kit (Invitrogen) as described in the instructions manual with minor modifications (Gottlieb et al. 2005). Selective primers were combined as in Table 2.

Table 2. AFLP Primers used in this study.

Primers	Sequence 5´-3´
E+AAC	GAC TGC GTA CCA ATT CAA C
E+ACC	GAC TGC GTA CCA ATT CAC C
M+A	GAT GAG TCC TGA GTA AA
M+G	GAT GAG TCC TGA GTA AG
M+C	GAT GAG TCC TGA GTA AC
M+T	GAT GAG TCC TGA GTA AT

E = *EcoRI*; M = *MseI*.

All PCR amplifications were performed in a TECHNE PROGENE thermal cycler.

Polyacrylamide gel electrophoresis conditions followed Gottlieb et al. (2005). A 30–330 bp AFLP® DNA Ladder (Invitrogen) size marker was included twice in each electrophoresis. Thus, the size of AFLP bands scored ranged from 90 to 330 bp. AFLP bands were visualized using the SILVER SEQUENCE ™ DNA Sequencing System (Promega). Air-dried gels were digitalized and visually analyzed using the Adobe Photoshop TM (Adobe Systems, Mountain View, Ca, USA).

Statistical methods

Each AFLP band was considered as a dominant allele at a unique locus. Table 3 shows the total number of AFLP bands, species-specific bands, and polymorphic and monomorphic bands detected for each selective primer combination from each fungal species and percentage of polymorphic loci (P%). Monomorphic bands (bands present in all individuals of a species) were discriminated within each species and across the entire data set. The binary matrix was analyzed with the program NTSYS-PC version 2.02 (Rohlf 1993). The unweighted pair-group arithmetic mean method (UPGMA) cluster analysis was performed based on the simple matching (SM), association coefficient (Sneath & Sokal 1973). The phenogram showing similarity relations was generated by the same program. The distortion produced during the grouping analysis was calculated using the correlation cophenetic coefficients (r) (Sokal & Rohlf 1962) using the NTSYS-PC version 2.02 (Rohlf 1993) program. A three-dimensional graphic was obtained with the principal coordinated ordination method (Gower 1966).

The percentage of polymorphic loci (P = number of polymorphic loci/ number of loci analyzed) × 100 for each primer combination was calculated.

Results

Characterization of AFLP markers

A total of 895 fragments in a range of 90 to 330 bp were generated for five *Saccobolus* species using six selective primer combinations. As a result, the number of reliable bands scored varied from 22 to more than 60, depending on the primer pair combination used.

Monomorphic AFLP band patterns were identified within each species. The diagnostic fingerprints (species-specific bands) of *Saccobolus citrinus,*

S. saccoboloides, *S. pseudodepauperatus* and *S. verrucisporus* were obtained and are described in Table 3.

Table 3. Total number of AFLP bands, species-specific bands (%), polymorphic and monomorphic bands detected for each selective primer combination in each fungal species assayed and percentage of polymorphic loci (P%).

Primer combination	Species	Bands				P%
		total no.	Polymorphic	Monomorphic	Species-specific %	
M+G/E+ACG						
	S. citrinus	26	2	24	26.9	7.7
	S. saccoboloides	32	1	31	12.5	3.1
	S. pseudodepauperatus	67	47	20	10.4	70.0
	S. verrucisporus	32	4	28	*	12.5
	S. versicolor	30	2	28	*	6.7
M+C/E+AAG						
	S. citrinus	30	2	28	36.6	6.7
	S. saccoboloides	33	1	33	39	3.0
	S. pseudodepauperatus	49	39	10	10	79.6
	S. verrucisporus	26	1	25	*	3.8
	S. versicolor	18	1	17	*	5.6
M+T/E+ACG						
	S. citrinus	57	27	30	22.8	47.4
	S. saccoboloides	58	13	45	27.6	22.4
	S. pseudodepauperatus	86	62	24	4.5	72.1
	S. verrucisporus	71	30	41	*	42.2
	S. versicolor	51	5	46	*	9.8
M+A/E+ACG						
	S. citrinus	66	36	30	4.5	54.5
	S. saccoboloides	72	11	61	12.5	15.3
	S. pseudodepauperatus	114	74	40	4.4	64.9
	S. verrucisporus	69	35	34	*	50.7
	S. versicolor	52	11	41	*	21.1
M+C/E+ACG						
	S. citrinus	68	34	34	17.6	50.0
	S. saccoboloides	69	20	49	15.9	29.0
	S. pseudodepauperatus	104	94	10	21.9	90.4
	S. verrucisporus	52	20	32	*	38.5
	S. versicolor	47	11	36	*	23.4
M+A/E+AAG						
	S. citrinus	72	30	42	6.9	41.7
	S. saccoboloides	86	27	59	10.5	31.4
	S. pseudodepauperatus	149	108	41	1.34	72.5
	S. verrucisporus	72	28	44	*	38.9
	S. versicolor	61	29	32	*	47.5

* Data not shown (see Table 4).

Primers MA/EAAG and M/EACG were more effective in detecting polymorphisms (Table 3). Genetic variability within species, measured in terms of percentage of polymorphic loci was variable (P = 83.8% for *S. pseudodepauperatus,* P = 41.0% for *S. citrinus*, P = 20.8% for *S. saccoboloides*, P = 22.8% for *S. versicolor*, and P = 39.8% for *S. verrucisporus*); i.e.,

S. pseudodepauperatus was the most variable species, and *S. saccoboloides* and *S. versicolor* the least variable.

Analysis of six samples from Gualeguaychú identified as *S. versicolor* using morphological characters displayed a similar diagnostic fingerprint to that of *S. verrucisporus* with all primer combinations used as is shown by the number of shared bands and the species-specific bands obtained between these two species (Table 4). Of the 330 bands obtained for *S. verrucisporus* and *S. versicolor*, 253 (77%) were shared between them and 54 were species-specific bands/diagnostic bands (present in both species). Of the 259 bands present in *S. versicolor*, only 6 were absent from *S. verrucisporus*.

Table 4. Total number of AFLP bands, species-specific bands and shared bands detected for each selective primer combination between *Saccobolus versicolor* and *S. verrucisporus* species.

Primer combination	Species	Total N° of bands	Species-specific Bands	Shared Bands
M+G/E+ACG	*S. verrucisporus*	32	10	28
	S. versicolor	30		
M+C/E+AAG	*S. verrucisporus*	26	7	18
	S. versicolor	18		
M+T/E+ACG	*S. verrucisporus*	71	10	49
	S. versicolor	51		
M+A/E+ACG	*S. verrucisporus*	69	5	52
	S. versicolor	52		
M+C/E+ACG	*S. verrucisporus*	52	11	46
	S. versicolor	47		
M+A/E+AAG	*S. verrucisporus*	72	11	60
	S. versicolor	61		

Degree of similarity and cluster analysis

The degree of similarity among species was low. Between *S. citrinus* and the group formed by *S. verrucisporus* and *S. versicolor* (S = 0.57) and between these cluster and *S. pseudodepauperatus* (S = 0.56). The group formed by the isolates identified as *S. saccoboloides* clustered with the other three species with the lowest coefficient of similarity (S = 0.53). The phenogram obtained by means of UPGMA method (Fig. 1) showed that all the strains were grouped into four clusters. Little distortion occurred while constructing this phenogram, as implied by the value of the correlation cophenetic coefficients (r = 0.9846).

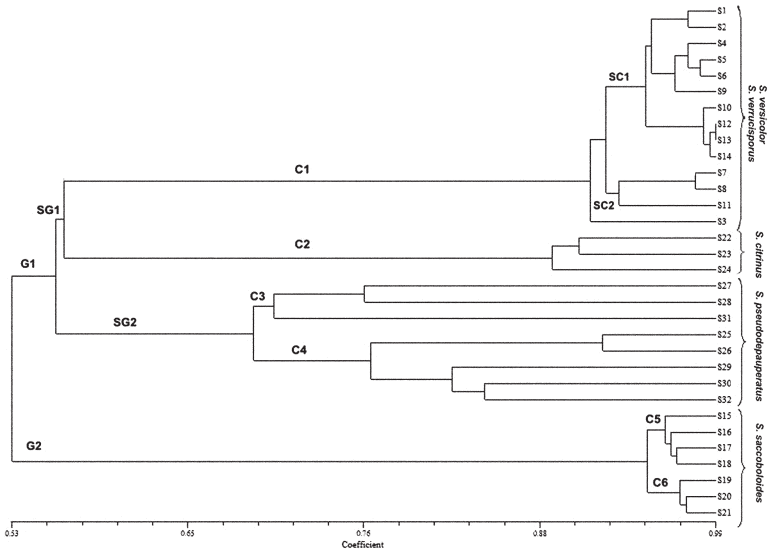

Figure 1. UPGMA phenogram showing relationships of *Saccobolus* species on the basis of simple matching (SM) association coefficient estimated from AFLP loci.

The main group (Fig. 1, group G1) comprised two subgroups (SG1 and SG2). Subgroup SG1 is formed by two sets (C1 and C2). The set C1 included all isolates of *S. verrucisporus* and *S. versicolor* and set C2, the three isolates identified as *S. citrinus*. The other Subgroup (SG2) comprised two sets, C3 and C4, formed by all isolates of *S. pseudodepauperatus.*

A second group (Fig. 1, G2) included two sets, C5 and C6, that included all isolates of *S. saccoboloides.*

Grouping analysis revealed that 14 isolates identified as *S. verrucisporus* and as *S. versicolor* clustered in one nucleus (Fig. 1, C1) with an origin of ramification in 0.92.

The set C1 is formed by two subsets (SC1, SC2) and one isolate S3 identified as *S. verrucisporus* separated from the rest. Only two pairs of isolates presented 100% of similarity (association coefficient 1.0), and they belonged to *S. verrucisporus*.

The ordination of isolates through the principal coordinated method let us distinguish four groups (Fig. 2, groups 1–4) in three-dimensional dispersion and showed nearly the same relations between isolates as the phenogram (Fig. 1). The first group included all isolates of *S. saccoboloides*, very closely attached in the three axes. The second set showed *S. pseudodepauperatus* isolates differentiated in axis 1 but very closely in the other two axes. The third

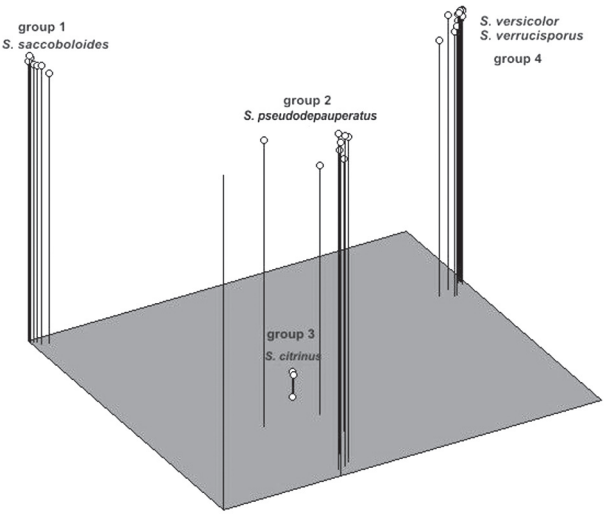

Figure 2. Three-dimensional graphic showing relationships of *Saccobolus* species obtained with Principal Coordinates ordination technique based on simple matching (SM) association coefficient estimated from AFLP loci.

comprised the three isolates of *S. citrinus*. The fourth and last group included the fourteen isolates identified as *S. verrucisporus* and as *S. versicolor* joined closely together in the three axes, thus revealing a high degree of similarity.

Discussion

There are many traits that are used in the traditional and modern mycology that contribute to taxonomical studies of fungi. These include morphological, anatomical, biochemical, and DNA based methods. However, fungal species descriptions are based mainly on morphological characters (Brasier 1997). Although identification of *Saccobolus* species was possible, coinciding morphological, cytological, and developmental characters make it difficult to achieve a clear delimitation. Previous isoenzymatic studies (Ramos et al. 2000) showed a low intraspecific variability and characterized the *Saccobolus* species by some isoenzymatic bands.

In this study a high number of AFLP bands were analyzed, which confirmed earlier results. The phenogram and ordination analysis both showed the same four clearly separated clusters, three of them corresponding to each of the three species identified as *S. citrinus*, *S. saccoboloides,* and *S. pseudodepauperatus*. Isolates identified as *S. versicolor* using morphological characters shared almost 97.7% of AFLP bands with *S. verrucisporus* in all primer combinations tested and joined together in the three axes of the ordination graphic and clustered

very closely in the phenogram, suggesting that isolates previously identified as *S. versicolor* could represent an intraspecific variant of *S. verrucisporus*. A higher number of isolates will need to be assessed in order to resolve this relationship.

Previous morphological and isoenzymatic studies in *Saccobolus* (Ranalli & Mercuri 1995, Ramos et al. 2000) indicated low intraspecific variability. A high number of AFLP bands obtained with the six primers allowed us to detect intraspecific variability.

The AFLP technique permits differentiation of *Saccobolus* species by providing a greater number of species-specific bands. This result coincides with previous isozyme studies (Ramos et al. 2000), where ALP (alkaline phosphatase) was the only system that showed a diagnostic electromorph for each species. Moreover, AFLPs are better able to differentiate species and detect intraspecific variability than isozymes because they allow extensive genome sampling and provide a great number of DNA markers and therefore a higher number of species-specific markers.

This technique could become a powerful tool for problematical genera such as *Saccobolus*, in which overlapping characters make species identification difficult and where a high intraspecific homogeneity does not allow detection of variability using traditional taxonomic and isozyme methods.

Acknowledgments

This study was supported by a grant from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad de Buenos Aires, Argentina. We thank, Dr. Lina Bettucci and Dr. Sandra Lupo for the critical review of the manuscript.

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